Amycolatopsis kentuckyensis sp. nov., *Amycolatopsis lexingtonensis* sp. nov. and *Amycolatopsis pretoriensis* sp. nov., isolated from equine placentas

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Actinomycete strains isolated from lesions on equine placentas from two horses in Kentucky and one in South Africa were subjected to a polyphasic taxonomic study. Chemotaxonomic and morphological characteristics indicated that the isolates are members of the genus *Amycolatopsis*. On the basis of phylogenetic analysis of 16S rDNA sequences, the isolates are related most closely to *Amycolatopsis mediterranei*. Physiological characteristics of these strains indicated that they do not belong to *A. mediterranei* and DNA relatedness determinations confirmed that these strains represent three novel species of the genus *Amycolatopsis*, for which the names *Amycolatopsis kentuckyensis* (type strain, NRRL B-24129^T = LDDC 9447-99^T = DSM 44652^T), *Amycolatopsis lexingtonensis* (type strain, NRRL B-24131^T = LDDC 12275-99^T = DSM 44653^T) and *Amycolatopsis pretoriensis* (type strain, NRRL B-24133^T = ARC OV1 0181^T = DSM 44654^T) are proposed.

INTRODUCTION

Over the past decade, actinomycetes have been reported to be a significant emergent cause of placentitis and abortion in horses in Kentucky (Giles *et al.*, 1993; Hong *et al.*, 1993; Donahue & Williams, 2000). The term nocardioform placentitis has been used to describe this distinct type of placentitis in horses, in which lesions are observed on the chorionic surface of the placenta and Gram-positive branching micro-organisms are recovered upon culture. The condition was first diagnosed in 1986 at the University of Kentucky Livestock Disease Diagnostic Center, but this type of placentitis has only recently been confirmed to occur outside Kentucky. In Kentucky, at least three different groups of bacteria (all Gram-positive branching bacilli) have been associated with nocardioform placentitis. Most of the severe infections were caused by the recently described actinomycete *Crossiella equi* (Donahue *et al.*, 2002). A small number of strains isolated from placental lesions appear to be members of the genus *Streptomyces*, whereas additional strains identified as members of the genus *Amycolatopsis* have been isolated from placental lesions since the late 1980s. Two of these strains, isolated from lesions on placentas from mares in Kentucky during 1999, along with a strain isolated from an equine placenta in Pretoria, South Africa, during 2000 (Volkmann *et al.*, 2001), were characterized in a polyphasic taxonomic study.

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of *Amycolatopsis kentuckyensis* NRRL B-24129^T, *Amycolatopsis lexingtonensis* NRRL B-24131^T, *Amycolatopsis pretoriensis* NRRL B-24133^T and *Amycolatopsis mediterranei* NRRL B-3240^T are AY183357, AY183358, AY183356 and AY184424, respectively.

METHODS

Primary storage of strains was as lyophilized ampules of mycelial and spore suspensions in sterile beef serum held at 4 °C. Working stock cultures were maintained on slants of ATCC medium no. 172 (Cote *et al.*, 1984) and stored at 4 °C until needed. Gross morphological observations of colonial characteristics were made after 14 days on ATCC medium no. 172 (Cote *et al.*, 1984) and those described by Shirling & Gottlieb (1966) for the International *Streptomyces* Project.

Biomass for extraction of DNA was grown as 7-day streak cultures on ATCC medium no. 172 agar plates. Chemotaxonomic analysis of strains for fatty acids, cell-wall diamino acid and whole-cell sugars was performed on autoclaved biomass by using previously described methods (Staneck & Roberts, 1974; Grund & Kroppenstedt, 1989; Saddler *et al.*, 1991). Physiological tests were evaluated by using the media of Gordon *et al.* (1974). Allantoin hydrolysis was evaluated in the basal medium suggested by Gordon *et al.* (1974) for aesculin hydrolysis. Phosphatase activity was evaluated by using the method of Kurup & Schmitt (1973). Temperature range for growth was determined on slants of ATCC medium no. 172 agar (Cote *et al.*, 1984). Genomic DNA was isolated, purified and sequenced following previously described procedures (Labeda & Kroppenstedt, 2000). A phylogenetic tree was constructed within the ARB software environment for sequence data, developed by Wolfgang Ludwig and Oliver Strunk, Lehrstuhl für Mikrobiologie, University of Munich, Germany (http://www.arb-home.de), using evolutionary distances by the method of Kimura (1980) and linkages by the neighbour-joining method of Saitou & Nei (1987). Genomic DNA was isolated and DNA relatedness between *Amycolatopsis mediterranei* NRRL B-3240^T and the equine isolates was determined spectrophotometrically in duplicate as described previously (Labeda, 1998).

RESULTS AND DISCUSSION

All three strains were found to have *meso*-diaminopimelic acid as the diamino acid in hydrolysates and arabinose and galactose as the primary sugars in whole-cell hydrolysates. The predominant phospholipid was phosphatidylethanolamine with small amounts of phosphatidylmethylethanolamine, while the primary menaquinones were MK-9(H_2) and MK-9(H_4). These chemotaxonomic properties are very characteristic of members of the genus *Amycolatopsis*. Fatty acid methyl ester profiles of all three strains (Table 1) consisted of straight- and branched-chain fatty acids, also typical of *Amycolatopsis*. Moreover, these micro-organisms produce branching vegetative mycelium that fragments into ovoid rod-shaped arthrospores, which is typically observed for members of this genus. Morphological and chemotaxonomic properties of the strains are in good agreement with those described by Lechevalier *et al.* (1986), when the genus *Amycolatopsis* was first proposed.

The 16S rRNA gene sequences determined in this study for equine isolates NRRL B-24129^T, NRRL B-24131^T and NRRL B-24133^T and *A. mediterranei* NRRL B-3240^T have been deposited in GenBank under accession numbers AY183357, AY183358, AY183356 and AY184424, respectively. Phylogenetic analysis indicates that all of the equine isolates are closely related to *A. mediterranei* NRRL B-3240^T with a bootstrap value of 95 % by neighbour-joining analysis, as can be seen clearly in Fig. 1. Tree topographies from the maximum-parsimony and maximum-likelihood methods were very similar. The topographies strongly suggest that the strains represent individual species, with 16S rRNA gene sequence similarities between them ranging from 99·2 to 99·8 % and similarity to *A. mediterranei* NRRL B-3240^T ranging from 98·8 to 99·4 %. Determinations of DNA relatedness among these strains and with *A. mediterranei* NRRL B-3240^T, as shown in Table 2, confirm that these strains represent distinct novel species, based on whole-genomic DNA relatedness of significantly less than 70 % among all three equine strains and with *A. mediterranei* NRRL B-3240^T.

Table 1. Fatty acid content of equine *Amycolatopsis* species

Species: 1, *A. kentuckyensis* NRRL B-24129^T; 2, *A. lexingtonensis* NRRL B-24131^T; 3, *A. pretoriensis* NRRL B-24133^T. Values are percentages of total fatty acids present; minor components are not shown.

Fatty acid	1	2	3
iso-$C_{14:0}$	2·19	4·14	3·44
iso-$C_{15:0}$	14·15	10·18	7·91
anteiso-$C_{15:0}$	3·25	1·22	
$C_{15:1}$ B	0·99	1·66	3·313
$C_{15:0}$	5·13	4·21	3·11
iso-$C_{16:0}$	17·19	42·85	45·49
$C_{16:1}$ *cis* 9	2·26	4·03	
$C_{16:0}$	2·97	2·21	
$C_{16:0}$ 9? methyl	3·32	1·46	
iso-$C_{17:0}$	2·39	2·05	
anteiso-$C_{17:0}$	6·64	1·67	1·37
$C_{17:1}$ *cis* 9	10·06	7·75	8·86
$C_{16:0}$ 2-hydroxy	2·85	7·75	12·28
$C_{17:0}$	6·22	3·28	2·68
$C_{17:0}$ 10-methyl	5·29	1·83	2·58

Gross morphological characteristics and differential physiological properties of these strains (as shown in Table 3) are consistent with molecular systematic observations and demonstrate clearly that the strains are different from each other, as well as from other described species in the genus *Amycolatopsis*. It is proposed that the following novel species should be created with their respective type strains: *Amycolatopsis kentuckyensis* NRRL B-24129^T, *Amycolatopsis lexingtonensis* NRRL B-24131^T and *Amycolatopsis pretoriensis* NRRL B-24133^T. Formal descriptions of each of these species follow. Additional actinomycete strains isolated from equine placentas in Kentucky during the 1999 and 2002 breeding seasons are currently under study to determine if additional strains that are representative of these new taxa can be found, as well as additional novel species.

Description of *Amycolatopsis kentuckyensis* sp. nov.

Amycolatopsis kentuckyensis (ken.tuc.ky.en′sis. N.L. fem. adj. *kentuckyensis* from Kentucky, named after the place of origin, state of Kentucky, USA).

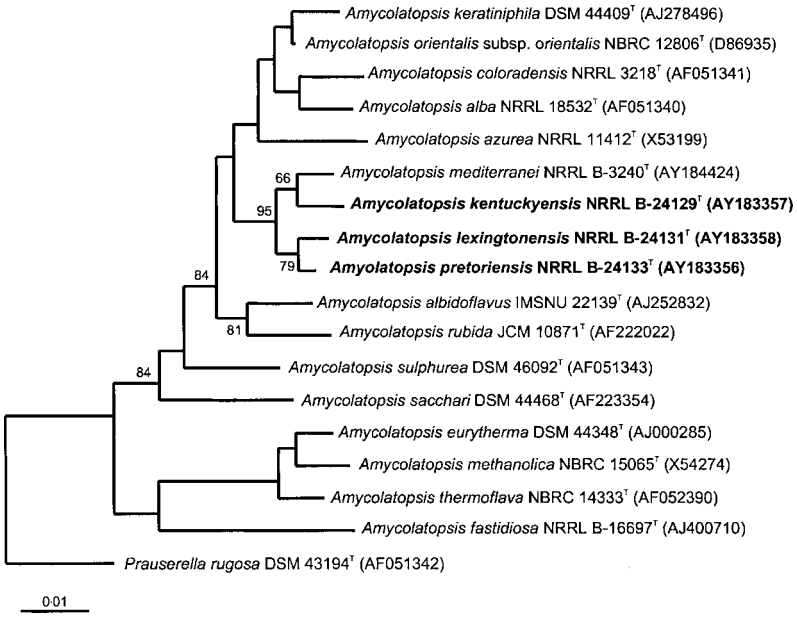

Fig. 1. Phylogenetic tree of the genus *Amycolatopsis*, calculated from 16S rDNA sequences by using Kimura's evolutionary distance method (Kimura, 1980) and the neighbour-joining method of Saitou & Nei (1987). Bootstrap values, expressed as percentages of 500 replications, are given at branch-points. Bar, 0·01 nucleotide substitutions per site.

Well-developed, yellow-orange to brownish-orange substrate mycelium is produced on most media. Aerial mycelium that ranges from light orangish-white to greyish orange-white in colour is produced on most media. A faint brownish soluble pigment is produced on some media. Chemotaxonomic characteristics are typical of the genus *Amycolatopsis*. Casein, aesculin, gelatin, hypoxanthine, tyrosine, urea and hippurate are hydrolysed or decomposed. Adenine, allantoin, starch and xanthine are not hydrolysed or decomposed. Nitrate is not reduced. Phosphatase is produced. Acetate and citrate are assimilated. Benzoate, lactate, malate, mucate, oxalate, propionate, succinate and DL-tartrate are assimilated weakly, if at all. Acid is produced from adonitol, arabinose, cellobiose, dextrin, dulcitol, D-fructose, D-galactose, D-glucose, glycerol, *myo*-inositol, lactose, maltose, D-mannose, melibiose, methyl α-D-glucoside, raffinose, rhamnose, salicin, D-sorbitol, sucrose and xylose. Acid is not produced from *meso*-erythritol, mannitol, melezitose or methyl β-xyloside. Grows in the presence of up to 5 % (w/v) NaCl. Temperature range for growth is 15–42 °C.

Table 2. DNA relatedness (%) between *A. mediterranei* NRRL B-3240^T and equine *Amycolatopsis* species

Strains: 1, *A. mediterranei* NRRL B-3240^T; 2, *A. kentuckyensis* NRRL B-24129^T; 3, *A. lexingtonensis* NRRL B-24131^T; 4, *A. pretoriensis* NRRL B-24133^T.

Strain	1	2	3
2	13·7		
3	21·5	42·1	
4	31·3	25·6	54·1

The type strain is NRRL B-24129^T (=LDDC 9447-99^T=DSM 44652^T). Isolated from an equine placenta in Lexington, Kentucky. Implicated in nocardioform placentitis in mares.

Description of *Amycolatopsis lexingtonensis* sp. nov.

Amycolatopsis lexingtonensis (lex.ing.ton.en′sis. N.L. fem. adj. *lexingtonensis* from Lexington, named after the place of origin, Lexington, Kentucky, USA).

Abundant dark orange-brown to dark reddish-brown substrate mycelium is produced on most media. Copious aerial mycelium is produced on most media, ranging in colour from light yellow to purplish-tan. A dark red to reddish-brown soluble pigment is produced on most media tested. Chemotaxonomic characteristics are typical of the genus *Amycolatopsis*. Casein, aesculin, gelatin, hypoxanthine, tyrosine, urea and hippurate are hydrolysed or decomposed. Adenine, allantoin, starch and xanthine are not hydrolysed or decomposed. Nitrate is reduced. Phosphatase is produced. Acetate, citrate, oxalate and propionate are assimilated. Benzoate, lactate, malate, mucate, succinate and DL-tartrate are not assimilated. Acid is produced from adonitol, arabinose, cellobiose, dextrin, D-fructose, D-galactose, D-glucose, glycerol, *myo*-inositol, maltose, D-mannose, melibiose, methyl α-D-glucoside, raffinose, rhamnose, salicin, sucrose and xylose. Acid is produced weakly from dulcitol, erythritol and mannitol. Acid is not produced from melezitose, methyl β-xyloside, sorbitol or trehalose. Grows in the presence of 5 % (w/v) NaCl. Temperature range for growth is 15–42 °C.

The type strain is NRRL B-24131^T (=LDDC 12275-99^T=DSM 44653^T). Isolated from an equine placenta in Kentucky.

Table 3. Differential properties of *A. kentuckyensis*, *A. lexingtonensis* and *A. pretoriensis* compared with previously described species of the genus *Amycolatopsis*

Taxa: 1, *A. kentuckyensis* NRRL B-14129^T; 2, *A. lexingtonensis* NRRL B-24131^T; 3, *A. pretoriensis* NRRL B-24133^T; 4, *A. mediterranei* NRRL B-3240^T; 5, *Amycolatopsis alba* NRRL 18532^T; 6, *Amycolatopsis albidoflavus* IMSNU 22139^T; 7, *Amycolatopsis azurea* NRRL 11412^T; 8, *Amycolatopsis coloradensis* NRRL 3218^T; 9, *Amycolatopsis eurytherma* DSM 44348^T; 10, *Amycolatopsis fastidiosa* NRRL B-16697^T; 11, *Amycolatopsis japonica* DSM 44213^T; 12, *Amycolatopsis methanolica* NBRC 15065^T; 13, *Amycolatopsis orientalis* subsp. *orientalis* NRRL 2450^T; 14, *Amycolatopsis rubida* JCM 10871^T; 15, *Amycolatopsis sacchari* DSM 44468^T; 16, *Amycolatopsis sulphurea* DSM 46092^T; 17, *Amycolatopsis thermoflava* NBRC 14333^T. +, Positive; −, negative; w, weak reaction.

Property	1	2	3	4	5*	6*	7*	8	9*	10*	11*	12*	13	14*	15*	16*	17*
Decomposition of:																	
Allantoin	−	−	−	−	+	+	−	−	+	−	+	+	−	−	+	−	+
Casein	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+
Aesculin	+	+	+	+	+	+	+	+	−	−	+	w	+	+	+	+	+
Gelatin	+	+	+	+	+	+	+	+	+	−	+	+	+	w	+	+	−
Hypoxanthine	+	+	+	+	+	+	+	+	w	−	+	+	+	+	−	−	+
Starch	−	−	−	−	+	−	−	+	−	−	+	−	+	−	−	−	−
Urea	+	+	w	+	+	w	+	−	+	+	−	−	+	+	+	−	+
Xanthine	−	−	−	−	+	+	−	−	−	−	+	−	+	+	+	−	+
Production of:																	
Soluble pigments	+	+	+	−	−	−	+	+	−	+	−	−	−	+	−	−	+
Nitrate reductase	−	+	−	−	−	+	+	+	+	+	−	+	+	+	+	+	−
Acid from:																	
Adonitol	+	+	−	−	+	+	+	−	+	−	+	+	+	+	+	−	+
Arabinose	+	+	+	+	+	+	+	−	+	w	+	−	+	+	+	−	+
Cellobiose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+
Dextrin	+	+	+	+	+	−	+	+	w	w	+	−	+	−	+	+	−
Erythritol	−	w	+	−	+	+	+	−	+	−	+	+	+	+	+	−	+
Fructose	+	+	+	+	+	+	+	+	+	w	+	+	+	+	+	+	+
Galactose	+	+	+	+	+	+	+	+	+	w	+	+	+	+	+	+	+
Inositol	+	+	+	+	+	+	+	+	+	−	+	−	+	+	−	−	−
Lactose	+	+	+	+	+	+	+	−	w	−	+	−	+	−	+	−	+
Maltose	+	+	+	+	+	+	+	+	−	w	+	+	+	−	+	+	−
Mannitol	−	w	−	+	+	w	+	+	+	−	+	+	+	+	+	+	+
Melibiose	+	+	+	+	+	−	+	−	−	−	+	−	−	−	−	−	+
Methyl α-D-glucoside	+	+	+	−	+	−	+	+	−	w	+	−	+	−	+	−	+
Raffinose	+	+	+	+	+	−	+	−	−	w	+	−	+	−	−	−	+
Rhamnose	+	+	+	+	−	−	−	−	+	−	−	+	+	+	+	−	−
Salicin	+	+	+	+	+	−	+	+	−	−	+	w	+	+	+	−	+
Sorbitol	+	−	w	w	−	−	−	−	+	−	−	+	−	−	−	−	+
Sucrose	+	+	+	+	+	+	+	+	−	w	+	+	+	+	+	+	−
Trehalose	+	−	+	+	w	+	+	+	+	w	+	+	+	+	+	+	+
Xylose	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	−	+
Growth in/at:																	
5% NaCl	+	+	+	w	−	+	+	+	+	−	w	+	w	+	+	−	+
45 °C	+	+	−	−	−	−	−	−	+	+	−	+	−	−	+	−	+

*Data from Kim *et al.* (2002).

Description of *Amycolatopsis pretoriensis* sp. nov.

Amycolatopsis pretoriensis (pre.tor.i.en′sis. N.L. fem. adj. *pretoriensis* from Pretoria, named after the place of origin, Pretoria, South Africa).

Well-developed greyish-yellow to orange-brown substrate mycelium is produced on most media. Abundant production of white to orange-white aerial mycelium occurs on most media. Faint soluble pigments are produced on some media, such as yeast extract/malt extract agar. Chemotaxonomic characteristics are typical of the genus *Amycolatopsis*. Casein, aesculin, gelatin, hypoxanthine and hippurate are hydrolysed or decomposed. Tyrosine and urea

are decomposed weakly. Adenine, allantoin, starch and xanthine are not hydrolysed or decomposed. Nitrate is not reduced. Phosphatase is produced. Acetate is assimilated. Benzoate, citrate, lactate, oxalate, propionate and succinate are assimilated weakly. Malate, mucate and tartrate are not assimilated. Acid is produced from arabinose, cellobiose, dextrin, dulcitol, erythritol, D-fructose, D-galactose, D-glucose, glycerol, *myo*-inositol, lactose, maltose, D-mannose, melibiose, methyl α-D-glucoside, raffinose, rhamnose, salicin, sucrose, trehalose and xylose. Acid is produced weakly from D-sorbitol. Acid is not produced from adonitol, mannitol, melezitose or methyl β-xyloside. Grows in the presence of 5 % (w/v) NaCl. Temperature range for growth is 15–37 °C.

The type strain is NRRL B-24133^T (=ARC OV1 0181^T=DSM 44654^T). Isolated from an equine placenta in Pretoria, South Africa.

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